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# Formulation and Characterization of Alginate Coated Chitosan Nanoparticles as Therapeutic Protein for Oral Delivery System

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**Abstract**

Nanoparticles have been promptly studied and developed for oral protein delivery. Selection of excipients and formulation method depends on the physicochemical characteristics of the protein carried. Therefore, this study aims to design a formulation and characterize the alginate coated chitosan nanoparticles which carried different protein. Alginate coated chitosan nanoparticles were formed using an ionic gelation method. Optimization was done by varying the parameter such as crosslinker concentration, agitation method, rate, and time. The results show that chitosan nanoparticles formed by sonication using sodium tripolyphosphate (STPP): chitosan (1:0.8) was the best method to form nanoparticles. The particle size and polydispersity index (PDI) of bovine serum albumin (BSA) and lysozyme nanoparticles were 812.2 nm and 0.412 for BSA while 793.3 nm and 0.438 for lysozyme. Encapsulation efficiency (EE) of BSA is 52 % and lysozyme is 68 %. *In vitro* tests on acidic/gastric conditions showed that lysozyme (±32 %) was released faster than BSA (±10 %) during the 24 h incubation. Under neutral/terminal intestine conditions, percentage of BSA (±17 %) release is slightly higher than lysozyme (±13 %) for 8 h incubation period. It was concluded that the formulation of alginate coated chitosan nanoparticles in this study appears to be more effective for BSA delivery than lysozyme.

**Key words**: Alginate, BSA, Chitosan, Lysozyme, Nanoparticle, Oral delivery

## Introduction

Therapeutic proteins have become an irreplaceable because of their vast benefits in the pharmaceutical industries [1]. Therapeutic proteins are used for both prevention and curation purposes by modulating the physiological or pathological processes of the target [2] and are proven effective in treating many potentially fatal diseases such as diabetes, heart problems, and cancer. Some proteins also have been effective as vaccines with the ability to stimulate the body's natural defense mechanism [3]. Since 1982 about 239 therapeutic proteins have been approved by the Food and Drug Association (FDA) [4]. Ye and Venkatraman (2019) [5] reported that currently more than 900 therapeutic proteins and peptides are undergoing various phases of clinical trials, including 330 monoclonal antibodies, 93 recombinant proteins, and 250 vaccines for numerous indications. Therapeutic proteins also offer more advantages compared with small molecules because of higher specificity, greater activity, and lower toxicity.

Most of the approved therapeutic proteins are formulated for parenteral administration, particularly for intravenous injection [5,6]. The intravenous route has drawbacks related to convenience and requirement of trained healthcare professionals for injection process. Moreover, protein based - vaccine that is injected through intramuscular and subcutaneous tissue has limitations in inducing mucosal immune responses [7]. Induction of mucosal immune responses on the surface of the digestive tract can be more easily done by oral administration [6].

Oral administration of therapeutic protein is the most convenient route, usually the safest, and least expensive [8]. However, it is challenging due to extreme environment in gastrointestinal tract. Therefore, the proteins need to be protected from that destructive condition and must be able to overcome multiple physicochemical and biological barriers found in the digestive tract.

Previous studies have been carried out to avoid protein degradation in the digestive tract, for instance, by using the characteristic of the protein itself. Proteins have amphoteric properties, so their surface charge vary depending on the pH of the solution [9]. The amphoteric character allows proteins to bind to other materials that have opposite charge at a certain pH. In the recent study, bovine serum albumin (BSA) and lysozyme were used as protein models. These proteins own different isoelectric points (pI). BSA is known to have pI 4.8, whereas lysozyme has pI of 10.5 - 11 [10,11].

Chitosan and alginate are natural polymers that have been approved by the Food and Drug Association (FDA) as non-toxic and safe (GRASS) [11-13]. They are widely used as excipient in the pharmaceutical industry and had been developed as drug carrier in the encapsulation process [15]. Chitosan has amino group that is soluble at acidic pH and insoluble at basic pH, while alginate is dissolved at basic pH and shrink under acidic pH. Simultaneous usage of these two polymers can protect proteins from degradation that might occur in the digestive tract. The amine group of chitosan interacts with the carboxylic group of alginate and forms a polyelectrolyte complex [16].

Chitosan has mucoadhesive properties that facilitate this particle to interact with the mucous layer in the digestive tract. Chitosan enables paracellular transport of a macromolecule by mediating the opening of strong bonds in the epithelial cells of digestive tract [17]. Alginate on the other hand is bio-adhesive, thus it is potential to increase drug residence time at resorption sites, which eventually improves the effectiveness and bioavailability of the drugs [18]. Chitosan and alginate also induce immune response in the body which is beneficial for oral vaccine delivery [11,18-20].

In regards to the formation of nanoparticles, the characteristics of the proteins affect the properties of the nanoparticles and their release profile. Since proteins are amphoteric, their surface charges depending on the pH of the solution wherein the protein is dissolved [9] and permit proteins to bind to other materials with different charges at a certain pH. Therefore, in this study, two proteins with different characters i.e pI which are BSA and lysozyme were used [10,11].

## Materials and methods

### Encapsulation of model protein alginate coated chitosan nanoparticles

#### *Preparation of chitosan nanoparticles*

Chitosan nanoparticles were prepared according to the previous method described by Borges *et al*. [19] with slight modifications. Chitosan was dissolved in 1 % acetic acid solution to final concentration 0.25 %. Chitosan solution was filtered to avoid the presence of undissolved particles. The chitosan-STPP nanoparticles were formed using three different methods: 1) sonication, 2) stirring, and 3) vortex. The obtained solution was centrifuged for 15 min at 9,500 rpm. The resulted pellets were collected in a 1.5 ml tube and then washed twice using an ultracentrifuge for 10 min at 30,000 rpm. Pellets were then stored in a freezer (−80 °C) for overnight prior to freeze-drying process. The dried particles were weighed before further use.

#### *Loading protein to chitosan nanoparticles*

Chitosan nanoparticles were re-dispersed in PBS 1× then disaggregated by ultrasonication. Protein models (BSA and lysozyme) were loaded into the chitosan nanoparticles upon mild agitation for 2 h. The solution of protein loaded nanoparticles was centrifuged for 10 min 30,000 rpm. Encapsulation efficiency (EE) and loading capacity (LC) of protein in chitosan nanoparticles were detected indirectly by determining the free protein remained in the supernatant using bicinchoninic acid (BCA) kit assay [22].

#### *Analysis of encapsulation efficiency (EE) and loading capacity (LC)*

The EE and LC were calculated by the following equation [23]:

$$\text{EE} = \frac{Total\ amount\ of\ protein - Free\ protein}{Total\ amount\ of\ protein}\ x\ 100$$

$$\text{LC} = \frac{Total\ amount\ of\ protein - Free\ protein}{Nanoparticles\ weight}\ x\ 100$$

#### *Preparation of alginate coated chitosan nanoparticles*

The protein that has been coated in chitosan nanoparticles were re-dispersed in phosphate buffered saline (PBS) 1×. The resulted solution was then slowly added to the sodium alginate solution (10 mg/ml)

upon mild agitation. The formed suspension was centrifuged for 10 min at 30,000 rpm and the supernatant was discarded. The pellets were dispersed back into calcium chloride ($CaCl_2$) solution (0.524 mmol/L) [22].

***Nanoparticles stability in acidic/neutral gastrointestinal-representing condition***

The pellets obtained from alginate coating were diluted using HCl 0.1 N pH 1.2. The solution was then incubated at 37 °C for 30, 60, 120 min and 24 h and then centrifuged at 30,000 rpm for 10 min to get the supernatant. The supernatant was tested for its protein content with the BCA kit assay. Stability in neutral condition was tested using PBS solution pH 7.4. The solution was incubated for 2, 4, 6 and 8 h.

**Nanoparticles characteristic**

***Analysis of nanoparticles size, PDI, and zeta potential***

Analysis of size, PDI, and particles zeta potential was conducted using particle size analyzer (PSA) (Malvern zetasizer, Nano ZS). The dual light scattering (DLS) technique was used in the determination of particle size and PDI.

***Fourier transform infrared (FTIR)***

IR-spectra were acquired using an attenuated total reflectance (ATR) FTIR-spectrometer 2 spectrum (Perkin Elmer, USA). Samples were dried by freeze-drying and then mixed with potassium bromide (KBr) powder. The analysis was performed with a resolution of 4000 - 400 $cm^{-1}$.

***Scanning electron microscopy (SEM)***

The surface morphology of the particle was observed using SEM JSM IT200 (JEOL, Japan). Samples were placed on a metal compartment then coated with gold for 5 min using a gold sputtering device ion coater iB2-Eiko and then observed under SEM at 15 kV [24].

**Results and discussion**

**Nanoparticle preparation and formation**

The chitosan/STPP nanoparticles were formed by three different methods, i.e using magnetic stirrer, vortex, and sonicator. In our first experiment, ratio of chitosan and STPP was 1:0.5. The nanoparticles formation using vortex and sonicator resulted relatively smaller size nanoparticles (< 300 nm) compared with that of using magnetic stirrer (> 600 nm). However, PDI of the nanoparticles produced by sonication and vortexing was high (> 0.5). Therefore, in our second experiment, chitosan: STPP ratio of 1:0.8 was also applied for both methods. Among those variations, chitosan: STPP ratio of 1:0.5 which was sonicated for 2 min at 20 A produced the smallest particle size (112.5 nm) with the lowest PDI (< 0.5).

A number of aspects contribute to the variation of particles size such as ionic strength between the materials used, material mixing method, stirring speed, chitosan, and crosslinker concentration ratio, chitosan deacetylation degree, and molecular weight of chitosan used, temperature and pH [25]. In addition to particles size, stirring process and ratio of chitosan-STPP also affected zeta potential. The higher the speed, the smaller the zeta potential value, while the concentration of STPP also affected the zeta potential value (**Table 1**).

The amount of STPP added affects the size of the particle [26]. It is known that excessive concentrations of STPP increases particles size because after a chitosan nanoparticle is completely formed, the unbound STPP attracts and links to more chitosan leading to form bigger particle. Moreover, the higher concentration of STPP decreases PDI [27]. Not only concentration of STPP, mixing speed also determines particles size and PDI. High mixing speed generates small particle size.

**Table 1** Effect of agitation method, speed, time, and ratio concentration between chitosan and STPP on the particle size of chitosan nanoparticles.

| Speed | Ratio C:S*) | Time | Particle size (nm) | Polydispersity index (PDI) | Zeta potential (mV) |
|---|---|---|---|---|---|
| | | | **Stirrer** | | |
| 500 rpm | 1:0.5 | 1 h | 453.3 ± 41.54 | 0.677 ± 0.074 | |
| 700 rpm | 1:0.5 | 1 h | 351.0 ± 36.50 | 0.818 ± 0.082 | |
| 1000 rpm | 1:0.5 | 1 h | 375.3 ± 35.23 | 0.695 ± 0.072 | 31.3 ± 0.85 |
| 1250 rpm | 1:0.5 | 1 h | 574.0 ± 105.5 | 0.668 ± 0.051 | |

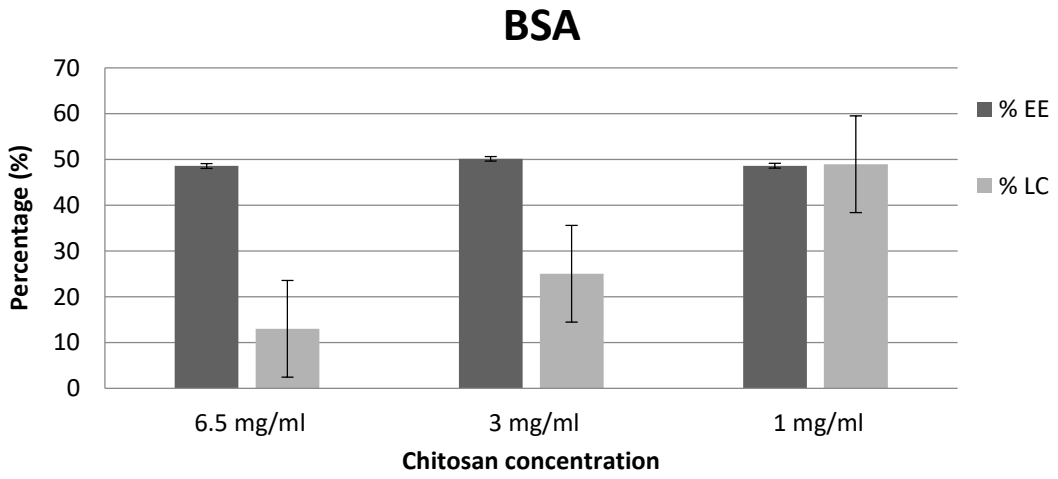
| Speed | Ratio C:S*) | Time | Particle size (nm) | Polydispersity index (PDI) | Zeta potential (mV) |
|---|---|---|---|---|---|
| | | | **Sonication** | | |
| 20 A | 1:0.5 | 2 min | 112.5 ± 1.41 | 0.317 ± 0.028 | 33.1 ± 0.36 |
| 20 A | 1:0.8 | 2 min | 271.2 ± 31.51 | 0.793 ± 0.181 | 19.4 ± 0.20 |
| 30 A | 1:0.5 | 2 min | 131.5 ± 1.30 | 0.277 ± 0.008 | |
| 30 A | 1:0.8 | 2 min | 210.4 ± 10.47 | 0.919 ± 0.091 | |
| | | | **Vortex** | | |
| 3000 rpm | 1:0.8 | 1 min | 151.7 ± 11.17 | 0.663 ± 0.168 | 20.6 ± 0.49 |
| 3000 rpm | 1:0.5 | 1 min | 257.7 ± 17.72 | 0.946 ± 0.077 | |

*) chitosan: STPP

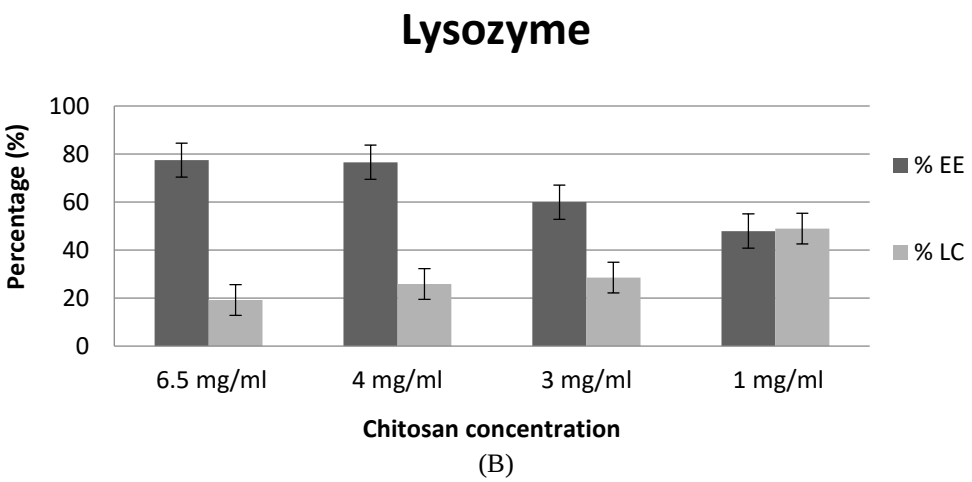
The formation of particles with an appropriate or sufficient speed accelerates the spread of crosslinking agents throughout the chitosan solution and increases the frictional force between the two materials to form monodisperse particles. An extremely high mixing is potential to damage interaction among particles which causes aggregation, meanwhile inadequate speed produces larger particles and polydisperse [28].

**BSA and lysozyme adsorption on chitosan nanoparticles**

Optimization of chitosan nanoparticle concentration was carried out to determine the most efficient formula to entrap 1 mg/ml BSA or lysozyme. This experiment used chitosan 6.5, 4, 3 and 1 mg/ml. The results showed that chitosan with a concentration of 6.5 mg/ml was not efficient. The lysozyme tested on chitosan 4 mg/ml showed EE that was almost the same as the EE in chitosan 6.5 mg/ml. In accordance with the data contained in the **Figure 1**, it can be seen that after the concentration of chitosan was reduced to 3 and 1 mg/ml, the EE decreases. EE describes the amount of protein entrapped in the particle while the LC describes the capacity of a nanoparticle to hold a protein with a certain concentration.




(A)




(B)

**Figure 1** Effect of chitosan nanoparticle concentration on the encapsulation efficiency (EE) and loading capacity (LC) of nanoparticles on BSA/lysozyme.

BSA entrapment was performed out using chitosan 6.5, 4, 3 and 1 mg/ml. BSA entrapment in chitosan 6.5 mg/ml showed EE of 48.5 %. The percentage of EE increased when the concentration of chitosan was reduced to 3 mg/ml but decreased when the concentration of chitosan was reduced to 1 mg/ml. The LC of both proteins tested shows that the smaller the concentration of chitosan nanoparticles used, the higher the LC. The low percentage of BSA trapped into nanoparticles can be influenced by several things including the pH of the adsorption media. One of the main factors that plays role in the association of proteins to nanoparticles is the electrostatic interaction between protein-polysaccharides. BSA is known to have pI at 4.8, so when the surrounding pH is higher than 4.8, BSA will be negatively charged. This property makes it easier for BSA to interact with the chitosan amine group which is positively charged and increases the efficiency of BSA entrapment into chitosan. Though in other study Calvo *et al.* [29] also concluded that in addition to electrostatic interactions, the binding between chitosan and BSA can also be influenced by other factors.

In the same context, Shen *et al.* [30] also explained that the electrostatic interaction between chitosan and BSA can decrease when pH > 4.7 because in that condition BSA is negatively charged and the amine group charge on chitosan is likely to move from positive at pH < 6.3 (pKa) to neutral or even negative charge when pH > 6.3. Sacco *et al.* [31] also wrote that chitosan can act as polyelectrolytes depending on the pH of the solution. This means that chitosan can interact with ions, small molecules, macromolecules that have opposite charges in the pH range 4 - 6 [32]. According to Zeng and Ruckenstein [9] the electrostatic interaction between chitosan microspheres and BSA (pI = 4.8) will be repulsive to pH > 6.5 but will become attractive at pH < 6.5. On contrary, the electrostatic interaction between chitosan microspheres and lysozyme (pI = 11) will be repulsive at pH < 6.5 and becomes attractive at pH > 6.5. On the other hand, the decreasing in entrapment when pH was shifted to 7 was explained by Katas *et al.*[33]. In their research using chitosan as a drug delivery system for hydrocortisone, they explained that the EE decreased when pH of the chitosan solution was raised from 3.0 to 7.0 may be occurred due to the decrease of the $-NH_3^+$group in the chitosan chain so that the binding efficiency of the chitosan and hydrocortisone bonds decreased. Torres *et al.*[34] reported that the adsorption capacity of BSA on crosslinked glutaraldehyde chitosan microspheres generally decreases when the pH of the solution is increased to pH 6.0 - 7.5. Lysozyme adsorption capacity on chitosan crosslinked glutaraldehyde microspheres, on the contrary, increased with increasing pH of the solution at 7.5 - 11. In addition, the adsorption capacity of chitosan to lysozyme might also be influenced by the natural affinity of lysozyme to the N-acetyl group in chitosan. Chitosan contains about 20 % chitin which has an N-acetyl-glucosamine unit in its structure [35]. In chitin, lysozyme is difficult to adsorbed at low pH because a decrease in pH can change the structure of lysozyme and affect its binding to the N-acetylglucosamine group [36].

**Nanoparticles stability in acidic-neutral gastrointestinal-representing condition**

Lysozyme and BSA are proteins with pI around 4.8 and 9.8 - 11. These proteins will be positively charged under conditions where the pH of the solution is below its pI (pH 1.2). This condition causes a high

percentage of lysozyme release under acidic conditions since 0 min (± 33 %). Under neutral conditions using PBS 1× solution pH 7.4, the release profile for both proteins were almost the same even though the release of BSA was still slightly higher. The amount of BSA released at 8 h of incubation was ±18 % while lysozyme was released ±13 % under similar incubation period. The stability of nanoparticles under acidic and neutral conditions shows different release profile. The percentage of lysozyme release from nanoparticles appears to be smaller than the percentage of BSA release. This condition seems to be influenced by nanoparticle electrostatic condition (charge) and the amount of protein entrapped [37].

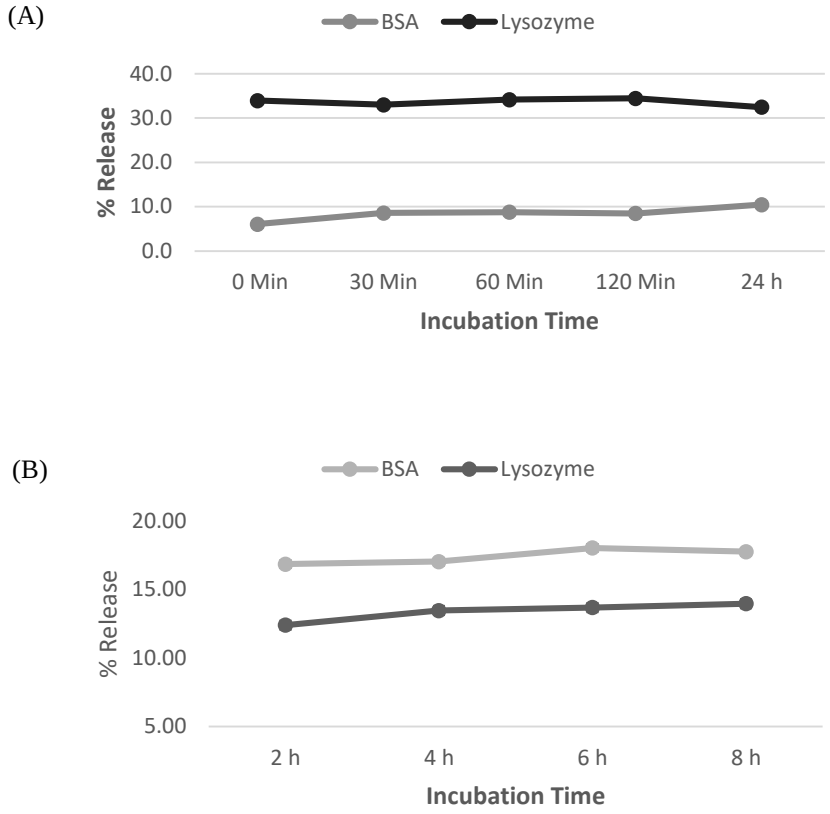



**Figure 2** Release profile of BSA and lysozyme from chitosan nanoparticles: (A) under acidic/gastric representing condition (0.1 N HCl pH 1.2), and (B) under neutral/terminal intestine representing condition (PBS 1× pH 7.4).

In acidic conditions (pH of 1.2), the release profile of BSA and lysozyme proteins is influenced by the condition of chitosan which is very soluble at low pH. At low pH, chitosan will be in the most positive condition because of the protonation of the free amino group of chitosan [38,39]. Lysozyme and BSA are proteins that were known to have pI at 4.8 and 9.8 -11. These proteins will also be positively charged under conditions where the pH of the solution is below its pI (pH 1.2). However, in BSA, the percentage of release under acidic conditions is quite low which is likely caused by the addition of alginate on the nanoparticles. This is supported by the result that shows the uncoated nanoparticle release BSA higher than the coated nanoparticle. Li *et al.* [22] has been reported in their research that alginate has the ability to protect BSA from degradation in acidic media and slow down the release process.

In addition, the fact that lysozyme has natural properties to degrade chitosan may also be a reason for the high release of lysozyme compared to BSA. Nwe *et al.* [40] reported that lysozyme is able to recognize the N-acetyl glucosamine group in chitosan molecules. The higher degree of acetylation in chitosan chain, the easier it is to degraded by lysozyme. Chitosan matrix which has a high degree of acetylation decomposes into monomers and oligomers after a few days exposed by lysozyme. Yomota *et al.* [41] also reported that acidic conditions can accelerate the degradation process compared to neutral conditions.

In general, there are many things that can affect the differences in releases activity of BSA and lysozyme. Borges *et al.* [38] wrote that the cause of the different release activities of chitosan nanoparticles can be caused by many things, including the preparation process of making the nanoparticles due to differences in the degree of deacetylation and molecular weight of chitosan used. Calvo *et al.* [29] reported that the rate of protein release can also depend on how much BSA is inserted into the nanoparticles, the higher the loading the faster the process of releasing BSA from within the nanoparticles.

**Characteristic of chitosan/alginate nanoparticles**

The characteristics of chitosan-alginate nanoparticles carrying BSA/lysozyme can be seen in **Table 2**. The particle size after the addition of BSA/lysozyme and alginate were 812.2 and 793.3 nm. This particle size was much different from the size of the empty chitosan nanoparticles, indicating that the addition of protein and alginate makes the size bigger.

**Table 2** Size of chitosan nanoparticles after the addition of protein and alginate at pH 7.4

| Sample | Particle size (nm) | Zeta potential (mV) | Polydispersity index |
|---|---|---|---|
| NP-BSA-ALG 4 mg pH 7.4 | 812.2 ± 5.93 | −18.83 ± 0.47 | 0.412 ± 0.102 |
| NP-LYS-ALG 4 mg pH 7.4 | 793.3 ± 11.58 | −25.0333 ± 5.01 | 0.438 ± 0.032 |

The surface charge of the nanoparticles was also analyzed by zetasizer (Malvern instrument). The data shows that all samples have a negative surface charge. This happens because the nanoparticles were coated with alginate on the outside.

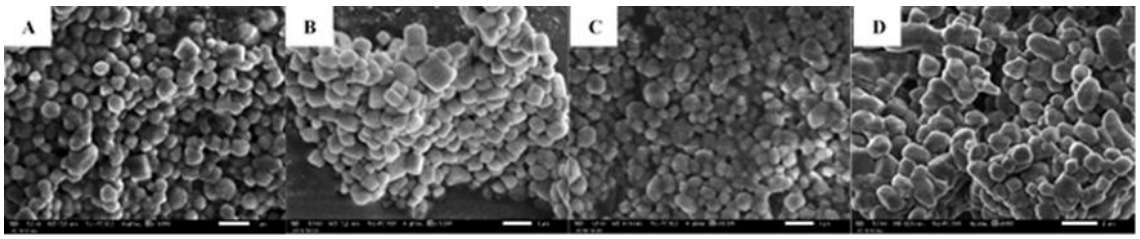
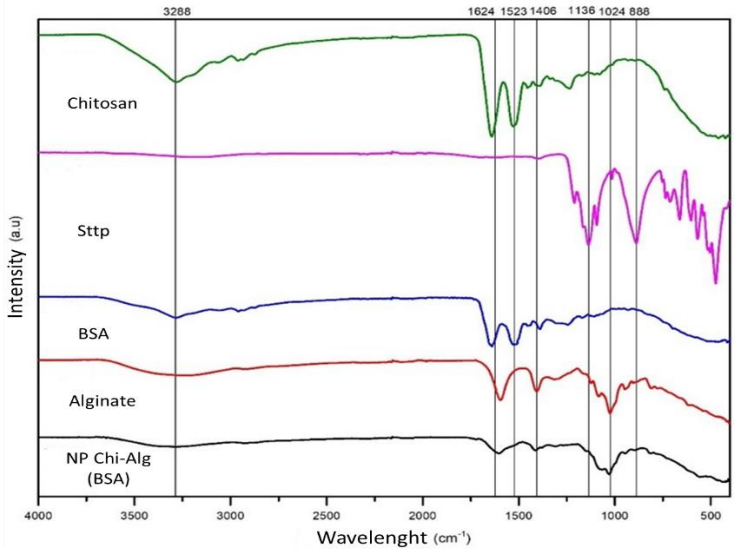



**Figure 3** SEM images of alginate-coated BSA-loaded chitosan nanoparticle: A) pH 7.4, B) pH 5.5, alginate-coated lysozyme-loaded chitosan nanoparticle: C) pH 7.4, and D) pH 8.5.

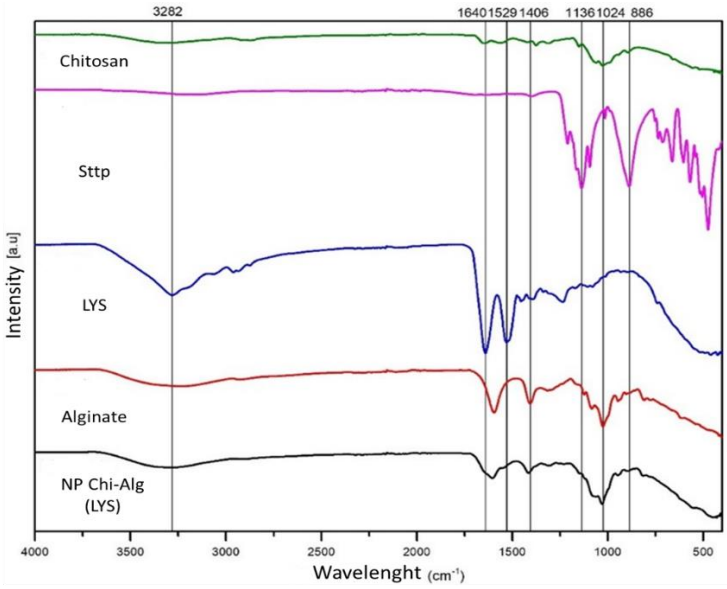
The morphology of the nanoparticles was observed with SEM. The observations showed that the majority of nanoparticles obtained were irregular spherical with a smooth surface and have a size below 1 µm. This confirms the measurement results previously by PSA.

**FTIR analysis**

The characteristic of each substance, including chitosan, STPP, sodium alginate, BSA/lysozyme, and alginate coated chitosan nanoparticles, were analyzed using FTIR. In chitosan spectra, the peak at 3288 $cm^{-1}$can be indicated as a vibrational strain of the hydroxyl group originating from chitosan. The strong and broad peaks in the chitosan spectra at 3400 - 3200 $cm^{-1}$ ranges correspond to combined peaks of O-H stretching and intermolecular hydrogen bonding [42]. The N-H stretching vibration of OH and $NH_2$ are overlapped in the same region [43]. The presence of absorption bands at 1645 $cm^{-1}$ originates from the vibrations of the carbonyl group which is very common in chitosan and also alginate. As a group of carbohydrate compounds, chitosan generally has a C-O bond in its chemical structure, this is evidenced by the presence of C-O strain absorption at 1028 $cm^{-1}$. This band can also indicate the presence of C-N which is commonly found in chitosan.




(A)




(B)

**Figure 4** FTIR spectra of nanoparticle: (A) alginate-coated BSA-loaded chitosan nanoparticle, and (B) alginate-coated lysozyme(LYS)-loaded chitosan nanoparticle.

FTIR analysis was also used to evaluate whether the encapsulation within chitosan nanoparticle had been successful. Spectral shift or minimal changes of FTIR spectrum would be attributed to possible chemical interaction between each parental compounds. It is expected that crosslinking of chitosan with STPP molecules would shift the peaks related to amide groups. Thus, comparing FTIR spectra of chitosan, STPP and chitosan nanoparticles in **Figure 4** reveals that the peak at 1645 $cm^{-1}$ in chitosan was shifted to 1602 $cm^{-1}$ in chitosan nanoparticles attributing to the occurrence of STPP molecules. In addition, the peaks at 1374 and 1028 $cm^{-1}$ in CS were shifted to 1411 and 1030 $cm^{-1}$ in chitosan nanoparticles, indicating that the amine groups of CS and polyanionic phosphate groups of STPP may take part in the reaction. Furthermore, the strong band at 1136 $cm^{-1}$ related to –COOH groups of TTP was not seen in the FTIR spectrum of chitosan nanoparticles, indicating that chitosan was completely crosslinked with STPP [14].

The –CO stretching vibration at 1645 $cm^{-1}$ in pure chitosan may also shifted to 1602 and 1604 $cm^{-1}$ after interaction of the amine group of chitosan with the carboxylic group of alginate. Further, the –

$NH_2$ bending peak at 1374 $cm^{-1}$ disappeared due to binding with alginate to form the nanoparticles [44,45]. Sodium alginate displayed two vibrations in the infrared spectrum due to the carboxylate group; an antisymmetric stretch at 1594 $cm^{-1}$ and a symmetric stretch at 1406 $cm^{-1}$. The peak at chitosan nanoparticle coated alginate showed vibration at 1604 $cm^{-1}$ (CS/ALG/BSA) and 1602 $cm^{-1}$ (CS/ALG/LYS) which may an asymmetric stretch originated from Sodium alginate as a carboxylate salt [46].

BSA and lysozyme are proteins which are composed of CH, NH, C = O, and C-N. In the IR spectrum of liquid samples, there are 3 or more absorption bands obtained, which is 3200, 1600 and 1200 $cm^{-1}$. The absorption bands in the 3200 $cm^{-1}$ indicate the presence of OH stretch vibrations originating from free OH in the structure of amino acids, or free carboxyl groups, and indicate the presence of NH groups. The widening band shows that there is an interaction of hydrogen bonds with water molecules. The presence of absorption bands at 1600 $cm^{-1}$ is thought to originate from the vibration vibrations of the carbonyl group which is very commonly found in proteins. Formed from peptide bonds, the C-N bond will be very common in its chemical structure, this is evidenced by the presence of strain absorption at 1200s $cm^{-1}$.

## Conclusions

BSA and lysozyme proteins were successfully encapsulated into alginate coated chitosan nanoparticles with different adsorption and release characteristics in each protein. The percentage of BSA adsorption of chitosan/alginate nanoparticles (52 %) was lower than the percentage of lysozyme adsorption (68 %). The percentage of lysozyme release was four times greater in acidic conditions than the percentage of BSA release. The release characteristics of the two proteins were not much different in alkaline conditions, ±17 % in BSA and 13 % in lysozyme.

Despite its success in encapsulated both protein (BSA/lysozyme), the formulation of this nanoparticle needs to be fully investigated in regard to its stability in human digestive track-representing *in vivo* study also to track the immunity response of body (protein uptake). Therefore, we expect that the following study will also analyze stability using simulated gastric fluid (SGF) and simulated colonic fluid (SCF). DC cell can be used as *in vitro* test to see effect of NP on DC cell maturation.

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